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THE UPTAKE OF NA-22 DURING INDUCTION IN PRESUMPTIVE EPIDERMIS CELLS OF THE RANA PIPIENS GASTRULA [1]

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Previous investigations (Barth, 1966) have shown that induction of various cell types from presumptive epidermis cells of the *Rana pipiens* gastrula by sucrose is a two-step process. The sucrose solution prepares the cells for induction by the salt solution in which they are cultured. Induction by the salt solution after sucrose treatment is proportional to the concentration of sodium chloride. The induction produced by lithium chloride in high concentrations for relatively short periods of time is likewise dependent upon the concentration of sodium chloride in the solution used for culturing the cells (Barth and Barth, unpublished data). While our standard solution contains other ions which may possibly complete the process of induction started by sucrose or lithium chloride, the sodium chloride dependency of the process suggested a study of the uptake of Na^{22}. Are the cells so altered by treatment with sucrose or lithium chloride that, upon return to standard solution, sodium ions enter more rapidly than in control cells which have not undergone treatment?

Methods

The procedure for isolating, treating and culturing small aggregates of cells from the gastrula has been described in earlier publications (Barth and Barth, 1967, for references). The concentration of NaCl in the standard solution used for operating is 515 mg. per 100 ml. For treatments of cells the medium was modified as described in detail in tables of data presented below. When aggregates were cultured in order to observe effects of treatments upon differentiation, the NaCl content of standard solution was 450 mg. per 100 ml.

An important aspect of the method for preparing presumptive epidermis cell aggregates is the use of Versene (EDTA) to loosen the superficial pigmented surface coat layer from the underlying presumptive epidermis cells. Only the latter were used in experiments, since the pigment coat layer of the anuran egg is relatively impermeable to most compounds.

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Most of the experiments reported below involved treatments of presumptive epidermis cells known to bring about induction of new cell types, followed by measurement of the influx of Na^{22}. The isotope was presented either together with the inductor (sucrose or the lithium ion), or following pre-treatment with the inductor. When the lithium ion was used as inductor, a positive correlation was observed between concentration of LiCl used for treatment and whether or not presumptive epidermis cells were induced to differentiate into a new cell type. Preliminary experiments indicated that both 2- and 4-hour treatment with LiCl at a concentration of 600 mg. per 100 ml. of standard solution (modified to contain no NaCl) induced extensive pigment ring cells, which are the precursors to melanocytes. When the concentration of LiCl was reduced to 300 mg., presumptive epidermis cells merely formed ciliated spheres of epidermal cells. Uptake of Na^{22} following 4 hrs. treatment in 300 mg. LiCl was only 214 counts per minute (c.p.m.) as compared with 355 at a concentration of 600 mg. LiCl per 100 ml. At 2 hrs. treatment the effect of increasing LiCl treatment on Na^{22} uptake gave values of 144 c.p.m. with 300 mg. LiCl and 260 c.p.m. following treatment with 600 mg. LiCl. These preliminary experiments indicated that induction of new types of differentiation, as well as uptake of Na^{22}, both were proportional to concentration of the lithium ion used as inductor. In experiments presented below, LiCl was used at concentrations known to induce new cell types.

Following exposure to the isotopes, aggregates were washed through three changes of unlabelled standard solution and pipetted, together with a minimal volume of solution, into aluminum planchets. Samples from the third wash solution were pipetted into other planchets to check for carry-over of isotope not bound to the cells. Values measured for the third wash were similar to the background c.p.m.

Planchets bearing the samples were dried in an oven at 110° C. for 30 min. and counted on a Model 186 Nuclear-Chicago low background counter. Counts per minute were calculated by averaging the 5-minute readings. Finally c.p.m. were expressed in terms of 200 aggregates, calculated from actual values read for samples consisting of 95 to 215 aggregates. Each aggregate consists of approximately 125 cells.

The isotopes used, obtained from New England Nuclear Corporation, were sodium^{22}, HCl solution, carrier-free (neutralized before use with $KHCO_3$), and uridine-2-C^{14} (specific activity 20–30 mC./m*M*). Sodium^{22} was diluted to a final concentration of 1.5 μC. per ml. in standard solution. Uridine-C^{14} was used at a concentration of 0.1 μC. per ml. of standard solution.

Results

1. Uptake of Na^{22} when sucrose is used as an inductor

Preliminary experiments showed that Na^{22} added to sucrose was taken up by the aggregates of presumptive epidermis. After the Na^{22} had reached an equilibrium value, the aggregates were returned to standard solution containing Na^{22} and the isotope left the aggregates.

When Ca^{++} was added to the sucrose the uptake of Na^{22} was reduced, as shown in Table I. The concentration of Ca^{++} was approximately that of our standard salt solution. Since Ca^{++} generally brings about a decrease in permeability of the cell

TABLE I

Effect of sucrose and of calcium on uptake of Na-22 by aggregates of presumptive epidermis cells

Concentrations of compounds were 58 mg./ml. sucrose; 0.2 mg./ml. $CaCl_2 \cdot 2\ H_2O$; and 1.5 μC. Na^{22}/ml.

Experiment No.	Compounds	Hours	No. of aggregates	c.p.m.	c.p.m. for 200 aggregates
1	Sucrose	1.5	177	3495	3950
	Sucrose + Ca	1.5	177	541	612
2	Sucrose + Ca	1.75	185	290	314
	Sucrose + Ca	3.75	185	304	329
3	Sucrose	0.5	185	650	703
	Sucrose	1.5	185	2148	2330

surface, the decreased uptake of Na^{22} appeared to be the result of a decrease in the permeability which was present in sucrose. The data in Table I could be best interpreted to demonstrate an increase in permeability of the aggregates in sucrose.

The next series of experiments was designed to determine whether the increased permeability caused by treatment with sucrose would lead to an increased uptake of Na^{22} after the aggregates were returned to standard solution. A comparison of Na^{22} uptake by aggregates which were treated with sucrose and by aggregates which were treated with standard solution is given in Table II. After treatment with sucrose and standard solution the aggregates are transferred to a solution containing Na^{22} and the uptake determined after varying periods of time. Sucrose-treated aggregates take up more Na^{22} than do control aggregates treated with standard salt solution containing 4.50 or 5.15 mg. NaCl per ml. Thus the sucrose treatment alters the cell surface so that Na^+ enters more rapidly. The largest differences between sucrose-treated aggregates and controls are found for the shorter periods of exposure to the isotope. Therefore the change in the cell surface produced by sucrose treatment may undergo recovery when the aggregates are transferred to standard salt solution. On the other hand, the sucrose-treated aggregates in Na^{22} may simply reach an equilibrium more rapidly. More information on this question will be found in the section dealing with Na^{22} uptake with Li^+ as the inductor.

2. Uptake of Na^{22} when LiCl is used as an inductor

Preliminary experiments showed an increase in uptake of Na^{22} with an increase in the concentration of LiCl. This suggested an alteration of the cell surface by LiCl. Since the induction by Li^+ is dependent upon the concentration of NaCl in the solution to which the Li^+-treated aggregates are returned, experiments were designed to measure the uptake of Na^{22} after Li^+ treatment.

Table III records the uptake of Na^{22} by aggregates which have been treated with lithium as compared with the uptake of controls. The results are similar to those recorded in Table II where sucrose is used as the inductor. More Na^{22} is taken up by those aggregates which have been exposed to lithium for 2.0 hours as compared with those which have been kept in standard solution for 2.0 hours.

TABLE II

Effect of sucrose pretreatment on uptake of Na-22 by presumptive epidermis cells

Concentrations of Na refer to mg. NaCl per 100 ml. standard solution.
Concentration of sucrose is 5.8 grams per 100 ml. glass-distilled water.

Exp. No.	Treatment		Post-treatment			No. of aggs.	c.p.m.	c.p.m. 200 aggs.	Difference ± sucrose
	Concentration	Hrs.	Conc.	Hrs.	μC. per ml.				
1	450 Na	1.5	450 Na	3.5	1.5	180	45	50	32
	Sucrose	1.5	450 Na	3.5	1.5	180	74	82	
2	450 Na	1.5	450 Na	1.25	1.5	155	35	45	44
	Sucrose	1.5	450 Na	1.25	1.5	155	69	89	
3	515 Na	1.5	257 Na	2.0	1.5	215	31	29	24
	Sucrose	1.5	257 Na	2.0	1.5	215	57	53	
4	515 Na	1.5	257 Na	0.5	1.5	145	37	51	54
	Sucrose	1.5	257 Na	0.5	1.5	145	76	105	
	515 Na	1.5	257 Na	1.5	1.5	116	44	76	5
	Sucrose	1.5	257 Na	1.5	1.5	116	47	81	
5	515 Na	1.5	450 Na	0.5	1.5	100	15	30	40
	Sucrose	1.5	450 Na	0.5	1.5	100	35	70	
	515 Na	1.5	450 Na	1.0	1.5	115	30	52	23
	Sucrose	1.5	450 Na	1.0	1.5	115	43	75	

In the next series of experiments (Table IV) the uptake of Na^{22} was measured during the period of lithium treatment. The NaCl content of the standard solution was lowered to 2.57 mg./ml., and 3.0 mg. of LiCl/ml. were added for induction. As a control, aggregates were kept in a solution containing NaCl at a concentration of 2.57 mg./ml. Both solutions contained Na^{22}. For periods ranging from 0.75 hr. to 5.3 hrs. more Na^{22} was taken up by those aggregates which were in the

TABLE III

Effect of lithium pretreatment on uptake of Na-22 by presumptive epidermis cells

Exp. No.	Treatment		Post-treatment			c.p.m.	No. of agg.	c.p.m. 200 agg.	Difference ± lithium
	Concentration	Hrs.	Conc.	Hrs.	μC. per ml.				
1	300 Li, 515 Na	2.1	450 Na	0.5	1.5	34	110	62	31
	515 Na	2.1	450 Na	0.5	1.5	17	110	31	
2	300 Li, 515 Na	2.1	450 Na	1.0	1.5	45	130	69	24
	515 Na	2.1	450 Na	1.0	1.5	29	130	45	
3	300 Li, 515 Na	2.0	257 Na	1.5	1.5	67	200	67	18
	515 Na	2.0	257 Na	1.5	1.5	49	200	49	

TABLE IV

Effect of lithium during uptake of Na-22 by presumptive epidermis cells

Exp. No.	Treatment		μC. per ml.	c.p.m.	No. of agg.	c.p.m. 200 agg.	Difference ± lithium
	Concentration	Hrs.					
1	300 Li, 257 Na	0.75	1.5	40	95	84	10
	257 Na	0.75	1.5	35	95	74	
2	300 Li, 257 Na	3.0	1.5	38	120	63	33
	257 Na	3.0	1.5	18	120	30	
	300 Li, 257 Na	5.3	1.5	40	115	70	25
	257 Na	5.3	1.5	26	115	45	
3	300 Li, 257 Na	2.0	1.5	50	120	84	29
	257 Na	2.0	1.5	33	120	55	
	300 Li, 257 Na	22.0	1.5	60	120	100	−370
	257 Na	22.0	1.5	282	120	470	
4	300 Li, 257 Na	20.5	1.5	87	125	139	−37
	257 Na	20.5	1.5	110	125	176	
	300 Li, 257 Na	24.5	1.5	85	120	142	−88
	257 Na	24.5	1.5	138	120	230	

solution containing LiCl (Table IV). It is during this period that induction occurs and after 5 hrs. the induction becomes independent of the concentration of NaCl in the culture medium.

For periods ranging from 20 to 24 hours the aggregates in lithium take up less Na^{22} that the controls. The significance of this difference is not apparent but it has no meaning for the induction process, which has occurred during the first 5 hrs. in LiCl. It is clear that the uptake of Na^{22} is higher than controls during and for some time after treatment with LiCl.

Another experiment was designed to determine the length of time, after LiCl treatment, during which the increased uptake could be detected. Thus aggregates were exposed as usual to LiCl and to standard solution. These aggregates then were transferred to standard solution for 1.0 hr. and 2.3 hrs. Aggregates were then exposed to Na^{22} for 30 minutes. Results showed an increased uptake of Na^{22} in LiCl-treated aggregates which had been in standard solution for 1 hr., but no increase over control values in those which had been in standard solution for 2.3 hrs. after LiCl treatment. Therefore the increased permeability produced by LiCl persists for about 1 hr. in standard solution. After this the permeability of LiCl-treated aggregates and control aggregates is about the same as measured by Na^{22} uptake.

In an effort to thrown some light upon the nature of the mechanism of the uptake of Na^{22} by LiCl-treated aggregates, two experiments in which the temperature was varied were carried out. Half the LiCl-treated aggregates were kept at 6° C. and half at 24° C. The Na^{22} uptake of each group was determined during

2.5 hrs. exposure to the isotope. The counts per minute at 6° were 68 and 69, respectively, and at 24°, 164 and 201. These values give a temperature coefficient, Q_{18°, of 2.6.

Discussion

A sequence of cell types beginning with radial nerve and ending with pigment cells can be induced in presumptive epidermis cells (Barth and Barth, 1967, for references). Two kinds of treatments have been found to induce this differentiation sequence: treatment of cells with the lithium ion; treatment with sucrose solutions.

The present experiments demonstrate that lithium and sucrose as inductors have several similarities in manner of action. Induction of new cell types after treatment with either lithium or sucrose is dependent upon the sodium concentration following treatment. In both cases uptake of Na^{22} by cells increases after treatment. The increased permeability indicated by this influx of Na^{22} returns to normal in from 1 to 2 hrs. Induction becomes independent of the sodium concentration in the medium about 2 hrs. after return of cells from inductor solution to standard solution.

A plausible explanation of these data would be that lithium and sucrose increase the permeability of the aggregates to sodium. When returned to a high concentration of sodium, the sodium ion penetrates more rapidly than in untreated controls. This phenomenon completes the process of induction, which becomes independent of the concentration of the sodium ion at about the same time as the permeability decreases to the normal value. This hypothesis, in the case of sucrose as an inductor, throws the entire responsibility for induction inside the cells upon sodium.

When lithium is used as inductor, short treatments with lithium require post-treatment with high concentrations of sodium (*i.e.*, induction is sodium-dependent). Longer treatments with lithium as inductor result in induction even at very low sodium concentrations in the medium used for culture. Therefore, lithium may act inside the cell in a manner similar to sodium, as well as by increasing the permeability of cell surfaces in a manner similar to sucrose treatment.

Further evidence of the action of the lithium ion inside the cell comes from experiments showing synergetic activity of lithium with sodium. For example, 300 mg. LiCl per 100 ml. with no sodium does not induce in 4 hrs. treatment; 300 mg. LiCl plus 257 mg. NaCl does induce in this time; 300 mg. LiCl with no sodium will induce with 20 hrs. exposure. Thus, given time, lithium can act like sodium inside the cell with respect to induction of new cell types.

In the case of sucrose treatment with no ions present, cells must be returned to a medium containing sufficient sodium to complete the induction. There is no evidence as yet that such induced cells show an increase in concentration of sodium over controls, however. This evidence could come only from determination of total sodium in sucrose-treated and untreated cells. Indeed, conceivably the sodium concentration after sucrose treatment could be less than controls, since there is no sodium in the external sucrose solution. The fact that more sodium enters after sucrose treatment may simply be due to a loss of sodium while the aggregates were in sucrose. Upon return to standard solution, cells would find a higher

concentration gradient between inside and outside and thus a more rapid penetration of sodium would take place.

This explanation would not hold, however, for the increased penetration of sodium after lithium treatment. Lithium is used in the presence of high sodium and thus loss of sodium from inside would not be expected during treatment. It seems clear that only determination of total sodium concentrations will clarify the situation and reveal whetther or not induction requires an increase in the absolute amount of sodium within the cell.

As regards permeability, we have used the term here although there is some question as to the validity of its use. For example, aggregates treated with sucrose or with lithium are irregular in shape while the controls round up and are spherical at the time of exposure to Na^{22}. Treated aggregates therefore have relatively more surface area, and would be expected to take up more Na^{22}. Our reason for using the phrase "permeability increase" is based upon experiments with uptake of Na^{22} in sucrose. When Ca^{++} is added to sucrose the uptake is reduced to one-sixth the value in sucrose alone. Since Ca^{++} usually reduces permeability of cell membranes we assume that it was acting in this capacity and that we are dealing with the phenomenon of permeability.

The data on temperature-dependence of Na^{22} uptake merit brief comment. A temperature coefficient for Q_{18° gave a value of only 2.6 for Na^{22} uptake. We can compare this value with the temperature coefficient, Q_{19° of 8.6, for uptake of ^{14}C-uridine (unpublished). Uridine enters sea urchin eggs by becoming phosphorylated (Piatigorsky and Whiteley, 1965). The results of our temperature studies show that a chemical reaction is involved in the case of uridine uptake by frog gastrula cells. The temperature coefficient for uptake of Na^{22} in frog gastrula cells is very low by comparison and possibly indicates that the penetration is dependent mainly upon diffusion.

Summary

1. The process of induction after sucrose treatment is accompanied by an increased uptake of Na^{22} as compared with the uptake by untreated controls.

2. The process of induction by lithium chloride treatment results in an increased uptake of Na^{22} during the lithium treatment.

3. The process of induction after lithium treatment is accompanied by an increased uptake of Na^{22} as compared with untreated controls.

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